JOURNAL OF VIROLOGY, Jan. 2009, p. 817–829 Vol. 83, No. 2
0022-538X/09/$08.00+0 doi:10.1128/JVI.01509-08

Classical Swine Fever Virus Can Remain Virulent after Specific Elimination of the Interferon Regulatory Factor 3-Degrading Function of N^{pro} ▽

Nicolas Ruggli,[1]* Artur Summerfield,[1] Ana R. Fiebach,[1] Laurence Guzylack-Piriou,[1] Oliver Bauhofer,[1]† Catherine G. Lamm,[1]‡ Sandro Waltersperger,[1] Keita Matsuno,[2] Luzia Liu,[1] Markus Gerber,[1] Kyung H. Choi,[3] Martin A. Hofmann,[1] Yoshihiro Sakoda,[2] and Jon-Duri Tratschin[1]

Institute of Virology and Immunoprophylaxis, Mittelhäusern, Switzerland[1]; Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan[2]; and Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch at Galveston, Galveston, Texas 77555-0647[3]

Received 17 July 2008/Accepted 21 October 2008

Pestiviruses prevent alpha/beta interferon (IFN-α/β) production by promoting proteasomal degradation of interferon regulatory factor 3 (IRF3) by means of the viral N^{pro} nonstructural protein. N^{pro} is also an autoprotease, and its amino-terminal coding sequence is involved in translation initiation. We previously showed with classical swine fever virus (CSFV) that deletion of the entire N^{pro} gene resulted in attenuation in pigs. In order to elaborate on the role of the N^{pro}-mediated IRF3 degradation in classical swine fever pathogenesis, we searched for minimal amino acid substitutions in N^{pro} that would specifically abrogate this function. Our mutational analyses showed that degradation of IRF3 and autoprotease activity are two independent but structurally overlapping functions of N^{pro}. We describe two mutations in N^{pro} that eliminate N^{pro}-mediated IRF3 degradation without affecting the autoprotease activity. We also show that the conserved standard sequence at these particular positions is essential for N^{pro} to interact with IRF3. Surprisingly, when these two mutations are introduced independently in the backbones of highly and moderately virulent CSFV, the resulting viruses are not attenuated, or are only partially attenuated, in 8- to 10-week-old pigs. This contrasts with the fact that these mutant viruses have lost the capacity to degrade IRF3 and to prevent IFN-α/β induction in porcine cell lines and monocyte-derived dendritic cells. Taken together, these results demonstrate that contrary to previous assumptions and to the case for other viral systems, impairment of IRF3-dependent IFN-α/β induction is not a prerequisite for CSFV virulence.

Interference with the innate immune response, particularly the alpha/beta interferon (IFN-α/β) system, has been considered a premise for the successful establishment of infection by an ever-increasing number of viruses (for selected recent reviews, see references 13, 16, and 34), including pestiviruses (1, 2, 7, 8, 33, 37, 43, 44). The strategies adopted by viruses to interfere with the primary immune response are extremely diverse. These strategies include suppression of IFN-α/β production, downregulation of IFN-α/β signaling, and inhibition of IFN-α/β-induced antiviral proteins. Most reports relate the in vivo consequences of a deletion, or inactivation of the gene or gene product interfering with innate immune defenses, to virus attenuation, irrespective of the pathway targeted by the virus (as exemplified in references 50 and 54).

Pestiviruses comprise classical swine fever virus (CSFV), a highly contagious pathogen of pigs (32); bovine viral diarrhea virus (BVDV), affecting cattle; and border disease virus, whose natural host is sheep. These animal viruses belong to the family *Flaviviridae* along with the flaviviruses and hepatitis C virus, which represent important human pathogens (11). CSFV is an enveloped virus carrying a single-stranded 12.3-kb RNA genome of positive polarity. The proteins encoded by the single open reading frame, flanked by 5′ and 3′ nontranslated regions (NTR), comprise four structural and eight nonstructural proteins (23). The first protein of the open reading frame is N^{pro}, an autoprotease that is present in all pestiviruses but not in the related flaviviruses and hepaciviruses. The N^{pro} protease autocatalytically generates its own carboxy terminus (38, 47, 52).

It is well documented that pestiviruses subvert innate immune defenses by preventing IFN-α/β induction, a property attributed to the viral N^{pro} protein (5, 9, 14, 17, 19, 22, 35, 37, 45). Several recent reports have shown that the N^{pro}s of both CSFV and BVDV interfere with IFN-β synthesis by inducing proteasomal degradation of IFN regulatory factor 3 (IRF3) (5, 9, 17, 22, 45). CSFV mutants lacking the complete N^{pro} gene (ΔN^{pro} CSFV) display growth characteristics similar to those of the respective wild-type viruses in porcine cell lines lacking the capacity for IFN-α/β synthesis. In contrast, replication of these N^{pro} mutants is impaired in IFN-α/β-producing cells such as porcine macrophages (37). Importantly, ΔN^{pro} CSFV mutants are attenuated in pigs (25, 49). Furthermore, replacement of the N^{pro} gene in a highly virulent CSFV by the corresponding gene of an avirulent vaccine strain resulted in a virus that was highly virulent (25). This indicated that the varying virulence observed between individual strains of CSFV

* Corresponding author. Mailing address: Institute of Virology and Immunoprophylaxis, Sensemattstrasse 293, CH-3147 Mittelhäusern, Switzerland. Phone: 41 31 848 9211. Fax: 41 31 848 9222. E-mail: nicolas.ruggli@ivi.admin.ch.

† Present address: Department of Molecular Virology, University of Heidelberg, D-69120 Heidelberg, Germany.

‡ Present address: Oklahoma Animal Disease Diagnostic Laboratory, Oklahoma State University, Stillwater, OK.

▽ Published ahead of print on 5 November 2008.

is not dependent on N^{pro}. All these observations led to the hypothesis that N^{pro} may have key functions in the pathogenesis of classical swine fever (CSF), by interfering with the IFN-α/β system rather than performing an essential function in replication per se (37, 49). However, the in vivo experiments with ΔN^{pro} CSFV did not determine if the attenuation of highly virulent CSFV after N^{pro} deletion was due solely to the loss of the N^{pro}-mediated block of IFN-α/β induction. The possibility remained that the lack of additional functions of N^{pro}, such as the protease function or a suboptimal RNA structure of the internal ribosomal entry site (IRES) due to the fusion of the 5′ NTR to the core (C) gene, was influential.

Therefore, the present study was aimed at specifically defining the role of the N^{pro}-mediated block of IRF3-dependent IFN-α/β induction in the pathogenesis of CSF. To this end, we searched for minimal amino acid changes that would inactivate the N^{pro}-mediated IRF3 degradation without affecting other potential functions of the N^{pro} protein or of the corresponding genomic region. Having demonstrated that IRF3 degradation was independent of the proteolytic activity of N^{pro}, we took advantage of rare CSFV strains reported to induce IFN-α/β in cell culture. Most pestiviruses will not induce IFN-α/β secretion in cell culture; in addition, they also inhibit the induction of IFN-α/β by strong viral inducers such as Newcastle disease virus (NDV) (35, 37). This was initially described as the "exaltation of NDV" (END) phenomenon, based on the observation that NDV replicates more efficiently in pestivirus-infected cells than in cells devoid of pestivirus infection (20, 21). The aforementioned rare CSFV strains that induce IFN-α/β in cell culture do not exhibit this END phenotype. Such strains were designated END negative (END^-) CSFV (39, 41, 46). Accordingly, we focused on amino acid residues occurring uniquely in the END^- CSFV strains GPE^-, Ames-END^-, and ALD-END^- to identify amino acid substitutions that would specifically eliminate the capacity of N^{pro} to degrade IRF3. Subsequently, we analyzed the effect of these mutations in CSFV infections in 8- to 10-week-old pigs to determine if this particular function of N^{pro} played a role in CSFV virulence.

MATERIALS AND METHODS

Cells. The swine kidney cell lines SK-6 (kindly provided by M. Pensaert, Faculty of Veterinary Medicine, Ghent, Belgium) and PK-15 (American Type Culture Collection [ATCC], Manassas, VA) were propagated in Earle's minimal essential medium (EMEM) and in Dulbecco's minimal essential medium supplemented with 7% horse serum (SVA, Hatunaholm, Sweden), respectively. HEK 293T cells (ATCC) were propagated in EMEM supplemented with 7% fetal bovine serum (Biochrom AG, Switzerland). The MDBK-t2 cell line was kindly provided by Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom) and maintained in EMEM supplemented with 7% fetal bovine serum and 10 μg/ml blasticidin (Invitrogen).

Viruses. CSFV strains vA187-1, vA187-ΔN^{pro}, vA187-ΔN^{pro}-Ub and vEy-37 were derived from the full-length cDNA clones pA187-1 (36), pA187-ΔN^{pro} (37), pA187-ΔN^{pro}-Ub (49), and pEy-37 (26), respectively. The vA187-1- and vEy-37-derived viruses with single (vA187-H_5Y, vA187-$L_{32}S$, vA187-$K_{61}N$, vA187-$C_{112}R$, vA187-$D_{136}N$, vEy-$C_{112}R$, and vEy-$D_{136}N$) or multiple (vA187-H_5Y-$L_{32}S$-$D_{136}N$) amino acid mutations in N^{pro} were generated from the corresponding mutant cDNA plasmids obtained by PCR-based site-directed mutagenesis with oligonucleotide primers containing the respective mutations and PfuUltra DNA polymerase (Stratagene), using previously described techniques (24). The subscript numbers refer to the amino acid position in the CSFV polyprotein. The bicistronic virus vA187-UbN^{pro}-EI-C was rescued from pA187-UbN^{pro}-EI-C, an infectious cDNA clone containing downstream of the viral 5′ NTR a cistron composed of the murine ubiquitin (Ub) gene fused to the N^{pro} gene followed by a stop codon, the encephalomyocarditis virus (EMCV) IRES, the C gene, and the remainder of the vA187-1 sequence. 5′ and 3′ truncations of the N^{pro} gene were introduced in the latter cDNA clone by PCR-based site-directed mutagenesis as described above, and the respective mutant bicistronic viruses were derived accordingly. The CSFV strain GPE^- was obtained from the Community Reference Laboratory for CSF, Institute of Virology, School of Veterinary Medicine, Hannover, Germany. The CSFV strain Ames-END^- was isolated from the Ames strain by the reverse plaque formation method (40, 41). The recombinant viruses vA187-N^{pro}(GPE^-) and vEy-N^{pro}(GPE^-) were generated from pA187-N^{pro}(GPE^-) and pEy-N^{pro}(GPE^-), obtained by replacement of the N^{pro} gene in pA187-1 and pEy-37, respectively, with the corresponding sequence of the GPE^- strain. All cDNA-derived viruses were rescued essentially as described elsewhere (30). Briefly, plasmid constructs were linearized at the SrfI site located at the end of the viral genomic cDNA sequence, and RNA was obtained by runoff transcription using the MEGAscript T7 kit (Ambion). After DNase I treatment and purification on S-400 HR Sephadex columns (GE Healthcare), RNA was quantified with a spectrophotometer and used to electroporate SK-6 cells.

Plasmid constructs. Plasmids pA187-1 (36) and pEy-37 (26), carrying the full-length genome of the Alfort/187 (GenBank accession number X87939) and Eystrup (GenBank accession number AF326963) strains, respectively, served as a basis for the construction of all mutant viruses described above. The nucleotide and amino acid numbers included in the present study refer to the sequences of the respective strains. All mutant constructs were generated using standard DNA cloning techniques. Mutagenesis was performed by PCR with PfuUltra DNA polymerase (Stratagene). Plasmids pCI-Flag-N^{pro} and pCI-N^{pro}-C*-EGFP are based on the pCI expression vector (Promega). pCI-Flag-N^{pro} encodes downstream of the AUG start codon the Flag epitope DYKDDDDK fused in frame to the N^{pro} gene of the Alfort/187 strain lacking the start codon. pCI-N^{pro}-C*-EGFP contains the N^{pro} gene of the Alfort/187 strain followed by the 5′-terminal 243 nucleotides of the C gene, a sequence encoding a GGSGGS linker, and the enhanced green fluorescent protein (EGFP) gene derived from pEGFP-N1 (Clontech). In the N^{pro} genes of plasmids pCI-Flag-N^{pro} and pCI-N^{pro}-C*-EGFP, the single amino acid substitutions H_5Y, $E_{22}V$, $L_{32}S$, $H_{49}L$, $K_{61}N$, $C_{69}S$, $C_{69}A$, $C_{112}R$, $C_{112}K$, $C_{112}S$, $C_{112}D$, $C_{112}A$, $D_{136}N$, $D_{136}E$, $D_{136}Q$, $D_{136}R$, $D_{136}V$; the amino acid deletions at position 112 (ΔC_{112}) and 136 (ΔD_{136}); and the 5′-terminal deletions resulting in the lack of 19, 22, and 27 amino acids at the amino terminus of N^{pro} (NΔ19, NΔ22, and NΔ27) were obtained by PCR-based site-directed mutagenesis as described above. The N^{pro} genes of strains GPE^- and Ames-END^- were amplified from RNAs of the respective viruses by reverse transcription-PCR using appropriate primers located in the 5′ NTR and C gene of the viral genome and cloned in pCR4-TOPO (Invitrogen) for GPE^- N^{pro} and in pGEM-T Easy (Promega) for Ames-END^- N^{pro}. The respective N^{pro} genes were then subcloned in pCI downstream of the Flag epitope in analogy to pCI-Flag-N^{pro} as described above. The $S_{32}L$ and $N_{136}D$ mutations in GPE^- N^{pro} and the $R_{112}C$ mutation in Ames-END^- N^{pro} were constructed as described above. For cytomegalovirus promoter-driven transcription of partially nontranslatable N^{pro} RNA, nucleotide 195 of the N^{pro} gene was deleted in pCI-Flag-N^{pro}, resulting in full-length N^{pro} RNA expressing only amino acids 2 to 74 of N^{pro} fused to the Flag tag. Plasmid pCI-Ub-Core was described before (35). For the mammalian two-hybrid assays, the porcine IRF3 gene (5) was introduced between the SgfI and PmeI sites of pFN10A(ACT) (Promega) in frame with the VP16 gene. The N^{pro} gene of the Alfort/187 strain and the mutants thereof encoding the $C_{69}A$, $C_{112}R$, and $D_{136}N$ substitutions were cloned into the SgfI and PmeI sites of pFN11A(BIND) (Promega) in frame with the yeast GAL4 gene. To this end, the respective genes were flanked with the sequences of the SgfI and PmeI restriction sites using standard PCR techniques as described above. The reporter plasmid p125Luc expressing firefly luciferase under the control of the IFN-β promoter (53) was a gift of Takashi Fujita, and phRL-SV40 constitutively expressing *Renilla* luciferase was obtained from Promega. Plasmids were propagated in *Escherichia coli* XL-1 blue cells (Stratagene) and purified using the NucleoBond plasmid DNA purification system (Macherey-Nagel). All newly synthesized and cloned DNA fragments were verified by nucleotide sequencing using the Thermo Sequenase DYEnamic direct cycle sequencing kit (GE Healthcare) and the Global IR^2 DNA sequencing system with e-Seq and AlignIR software (LI-COR Biosciences).

Immunoreagents. The E2-specific monoclonal antibody (MAb) HC/TC26 (15) was kindly provided by I. Greiser-Wilke (Hannover Veterinary School, Hannover, Germany). The rabbit polyclonal antisera against recombinant affinity-purified CSFV N^{pro} (35), CSFV C, and porcine IRF3 (5) were previously described. The anti-FLAG M2 MAb was purchased from Sigma. The MAb JL-8 (Clontech) was used for the detection of EGFP. Secondary antibodies Alexa Fluor 680 goat anti-rabbit immunoglobulin G (IgG) and IRDye 800 goat anti-mouse IgG were from Molecular Probes Inc., and Rockland Inc., respectively. The horseradish peroxidase-conjugated rabbit anti-mouse IgG was from Dako.

Virus titration. The virus titer, expressed in 50% tissue culture infective doses ($TCID_{50}$)/ml, was determined by end point dilution on SK-6 cells and immunoperoxidase staining using MAb HC/TC26 as described earlier (29).

SDS-PAGE and Western blotting. Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting essentially as previously described (35). Briefly, cells were lysed in a hypotonic buffer containing 20 mM morpholinepropanesulfonic acid, 10 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100 (pH 6.5), and 1 μl protease inhibitor cocktail (Sigma) per 10^6 cells to be lysed. After clarification by centrifugation at 15,000 × *g* for 10 min at 4°C, equal amounts of total protein were separated by SDS-PAGE under reducing conditions and transferred onto a 0.2-μm nitrocellulose membrane (Bio-Rad) using a Trans-Blot semidry transfer device (Bio-Rad). Membranes were blocked for 1 h at room temperature or overnight at 4°C with Odyssey blocking reagent (LI-COR Biosciences). For immunodetection, the membranes were incubated at 37°C with the respective primary and secondary antibodies for 30 min each in Odyssey blocking reagent. Between each incubation step, membranes were washed with phosphate-buffered saline supplemented with 0.1% Tween 20. After two final wash steps in the absence of detergent, the signal was acquired using the Odyssey infrared imaging system (LI-COR Biosciences).

Mammalian two-hybrid assay. Protein interactions were assayed with the CheckMate/Flexi Vector mammalian two-hybrid system (Promega). HEK 293T cells seeded at a density of 10^5 cells per well in a 24-well plate were transfected with a mixture of 100 ng/well each of pFN10A(ACT)- and pFN11A(BIND)-derived plasmid or control vector and 50 ng/well of the pGL4.31(*luc2P*/*GAL4*UAS/Hygro) reporter plasmid (Promega). The DNA transfections were performed with FuGENE 6 transfection reagent (Roche) according to the manufacturer's protocol with a 5:1 (vol/wt) ratio of FuGENE 6 to DNA. Cells were incubated for 48 h at 37°C prior to extraction and quantification of firefly and *Renilla* luciferase using the dual luciferase reporter assay system (Promega) and a Centro LB 960 luminometer with two automated injectors (Berthold Technologies). The individual firefly luciferase activities were normalized to the respective *Renilla* luciferase activity. The relative induction of firefly luciferase activity was then calculated based on the activity obtained with the empty vectors set to 1. Expression of the respective fusion proteins was analyzed by SDS-PAGE and Western blotting using the CSFV N^{pro}- and porcine IRF3-specific rabbit antisera described above.

Reporter gene assays. The reporter assay for IFN-β promoter activation was carried out as described before (35). Plasmid p125Luc served as a reporter for the inducible IFN-β promoter activity, and plasmid phRL-SV40 (Promega) was used for internal normalization. Briefly, HEK 293T cells were transfected with the expression and reporter plasmids using FuGENE 6 (Roche) as described above and challenged with poly(IC) transfection 24 h later. At 28 h after poly(IC) treatment, firefly and *Renilla* luciferase were quantified using the dual luciferase reporter assay system (Promega) and a Lumat LB 9507 or a Centro LB 960 luminometer with two automated injectors (Berthold Technologies), and the firefly luciferase values were normalized as described above.

IFN bioassay. The bioactivity of porcine IFN-α/β was assayed as previously described (35, 37) using the Mx/CAT reporter gene assay developed for the quantification of bovine IFN-α/β (12) and kindly provided by Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom). To this end, the culture supernatants and the porcine serum samples were added to the MDBK-t2 cells at appropriate dilutions allowing measurement in the linear range of the assay. Recombinant porcine IFN-α produced in HEK 293-EBNA cells (3) was used as a standard. Chloramphenicol acetyltransferase (CAT) was quantified using a CAT enzyme-linked immunosorbent assay (Roche). Infectious CSFV present in the samples was neutralized with polyclonal pig anti-CSFV serum, and poly(IC) was removed by RNase treatment as described previously (35).

Experimental infections of pigs. Specific-pathogen-free (SPF) pigs, 8 to 10 weeks old and with a body weight of approximately 30 to 40 kg, obtained from the breeding unit at the Institute of Virology and Immunoprophylaxis were infected via the oronasal route with a defined dose of virus (expressed in $TCID_{50}$ based on virus titration on SK-6 cells). Virus used for inoculation was diluted in 10 ml EMEM, and one half of the dose each was administered intranasally and orally. All inocula were titrated on SK-6 cells immediately before and after use to confirm the inoculation dose. For each virus to be tested, groups of three or four littermates were kept in separate isolation units. Body temperature and clinical score were monitored daily according to a defined scoring system as described previously (28). At daily intervals, whole blood was collected for serum preparation and EDTA-blood for white blood cell count and for preparation of leukocytes.

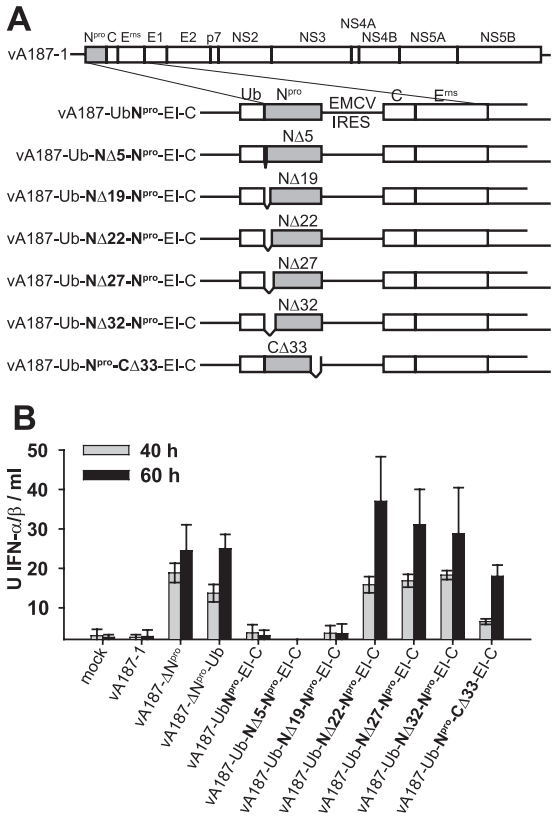

FIG. 1. The 19 amino-terminal amino acids of N^{pro} are dispensable for the block of IFN-α/β induction. (A) The RNA genomes of the parent CSFV and of the bicistronic constructs harboring sequential amino-terminal deletions of 5, 19, 22, 27, and 32 amino acids (NΔ5, NΔ19, NΔ22, NΔ27, and NΔ32) and a carboxy-terminal deletion of 33 amino acid (CΔ33) of the N^{pro} gene (gray box) are depicted schematically. For the bicistronic constructs, the murine Ub gene was inserted between the 5′ NTR and the N^{pro} gene, and the EMCV IRES (EI) was used to separate the N^{pro} gene from the C gene. (B) PK-15 cells were mock infected or infected at a multiplicity of infection of 2 $TCID_{50}$/cell with vA187-1, viruses lacking N^{pro} (ΔN^{pro} and ΔN^{pro}-Ub), or bicistronic viruses, as indicated on the *x* axis. The cells were cultured for 40 and 60 h. IFN-α/β activity was quantified in cell culture supernatants using the Mx/CAT reporter gene assay and recombinant porcine IFN-α. Error bars indicate standard deviations.

RESULTS

The 19 amino-terminal amino acids of CSFV N^{pro} are not required to block IFN-α/β induction. For BVDV, it was shown that nearly the entire N^{pro}, especially structures of the amino-terminal region, was critical for prevention of IFN-α/β induction (9, 14, 17). In order to determine if this was similar for the N^{pro} of CSFV, we constructed viruses expressing amino- and carboxy-terminally truncated N^{pro}. In the natural situation, N^{pro} generates the amino terminus of the C protein. Thus, the autoprotease function of N^{pro} is a prerequisite for the synthesis of viable virus. For analyzing N^{pro} deletion mutants independently of the autoprotease function of N^{pro}, we inserted an IRES sequence of the EMCV (EI) between the N^{pro} and the C genes, to obtain the bicistronic virus vA187-UbN^{pro}-EI-C (Fig. 1A). The mouse Ub gene was inserted upstream of the N^{pro} gene to avoid variations in the translation efficiency due to diverse nucleotide sequences when the N^{pro} gene was trun-

cated at its 5′ end. As expected, following infection of PK-15 cells, no IFN-α/β bioactivity was measured in the culture supernatant, whether monocistronic vA187-1 or bicistronic vA187-UbN^{pro}-EI-C virus carrying the entire N^{pro} gene was employed. This shows that N^{pro} can block IFN-α/β induction in the bicistronic context (Fig. 1B). Deletion of up to 19 amino acids at the amino terminus of N^{pro} did not abolish this function. However, truncation of at least 22 amino acids at the amino terminus or of 33 amino acids at the carboxy terminus of N^{pro} resulted in viruses that induced IFN-α/β similarly to viruses with deletion of the complete N^{pro} (vA187-ΔN^{pro} and vA187-ΔN^{pro}-Ub). This demonstrates that the functional domains of N^{pro} relating to inhibition of IFN-α/β induction are located downstream of the glycine residue at position 19 (G_{19}).

The autoproteolytic activity of N^{pro} is not required to block IFN-α/β induction. Residue E_{22} is involved in the proteolytic activity of N^{pro} (38). Considering that deletion of the amino-terminal 22 amino acids of N^{pro} abolishes the N^{pro}-mediated inhibition of IFN-α/β induction, this may suggest a requirement for a functional N^{pro} protease. For BVDV, there is good evidence that the proteolytic activity of N^{pro} is not required for the inhibition of IFN-α/β induction (9, 14, 17). In order to determine if this was also true for the N^{pro} of CSFV, we constructed plasmids for eukaryotic expression of a fusion polypeptide consisting of N^{pro}, carboxy-terminally truncated C, a flexible amino acid linker, and EGFP (N^{pro}-C*-EGFP) (Fig. 2A). The three residues E_{22}, H_{49}, and C_{69}, considered to form the active site of the autoprotease (38), were mutated individually or in combination. Based on the results obtained with the bicistronic viruses (Fig. 1), we also included expression constructs lacking codons for 19, 22, and 27 amino acids at the amino terminus (Fig. 2A). The polyprotein processing between N^{pro} and C of the parent and mutant N^{pro}-C*-EGFP was monitored in transient-expression experiments in HEK 293T (Fig. 2B) and SK-6 (data not shown) cells. In parallel, the same expression constructs were analyzed for their effect on poly(IC)-mediated IFN-β induction in HEK 293T cells. After mutagenesis of amino acids E_{22}, H_{49}, and C_{69}, cleavage was either partially (E_{22}V and C_{69}S) or completely (H_{49}L, C_{69}A, E_{22}V-H_{49}L, and E_{22}V-C_{69}S) abolished. Also, the deletion of 22 and 27, but not of 19, amino acids at the amino terminus of N^{pro} was sufficient to prevent its proteolytic activity (Fig. 2B). In agreement with the results obtained with the bicistronic viruses (Fig. 1B), N^{pro} lacking the 19 amino-terminal amino acids prevented poly(IC)-mediated activation of the IFN-β promoter, whereas constructs lacking 22 and 27 amino acids had lost this function (Fig. 2B).

These results indicate that amino acids 20 to 22 are crucial for the interference with IFN-β induction. Indeed, the E_{22}V substitution generated an N^{pro} that had completely lost its capacity to prevent IFN-β promoter activation. In contrast, the protease activity was still partially maintained (Fig. 2B). When C_{69} of the catalytic domain of the N^{pro} protease was mutated to serine or to alanine, the N^{pro}-mediated inhibition of the IFN-β promoter activation was not affected. With C_{69}S, the protease was still partially active, while with C_{69}A, there was no detectable proteolytic activity remaining (Fig. 2B). These data demonstrate that the absence of protease activity does not necessarily abolish the inhibition of the IFN-β promoter induction. Conversely, as described above, the E_{22}V mutation

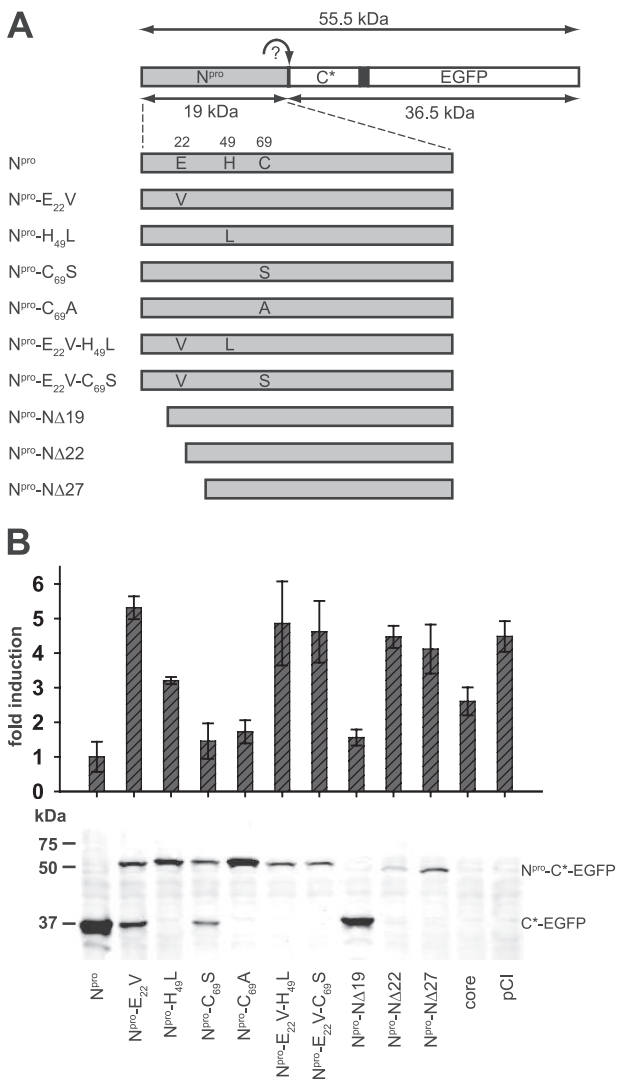

FIG. 2. The N^{pro}-mediated counteraction of IFN-α/β induction is independent of the autoproteolytic activity of N^{pro}. (A) The polyprotein expressed in the pCI vector under the control of a cytomegalovirus promoter and consisting of N^{pro}, carboxy-terminally truncated nucleocapsid protein (C*), a glycine-serine-rich amino acid linker (black box), and EGFP is represented schematically. The E_{22}V, H_{49}L, C_{69}S, and C_{69}A mutations, and combinations thereof, and the amino-terminal deletions of 19, 22, and 27 amino acids (NΔ19, NΔ22, and NΔ27) are also depicted. (B) HEK 293T cells (two replica wells each) were transfected with a mixture of the reporter plasmid p125Luc for IFN-β promoter activity, plasmid phRL-SV40 for internal normalization, and the respective expression plasmid indicated on the *x* axis. The plasmid pCI-Ub-Core encoding the CSFV nucleocapsid protein (core) and the empty vector (pCI) were used as controls. Cells of one well were then stimulated with poly(IC) transfection, and cells of the other well were mock transfected. The firefly luciferase activity was measured and normalized with constitutively expressed *Renilla* luciferase activity. Each bar represents the relative induction of poly(IC)-treated versus mock-treated cells. The results are the means from three independent sets of transfections, with error bars showing the standard deviations. Total protein was extracted from parallel wells, and the processing of the N^{pro}-C*-EGFP polyprotein was analyzed by Western blotting using an anti-EGFP MAb. The positions of the 55.5-kDa N^{pro}-C*-EGFP precursor polyprotein and of the 36.5-kDa C*-EGFP cleavage product are indicated.

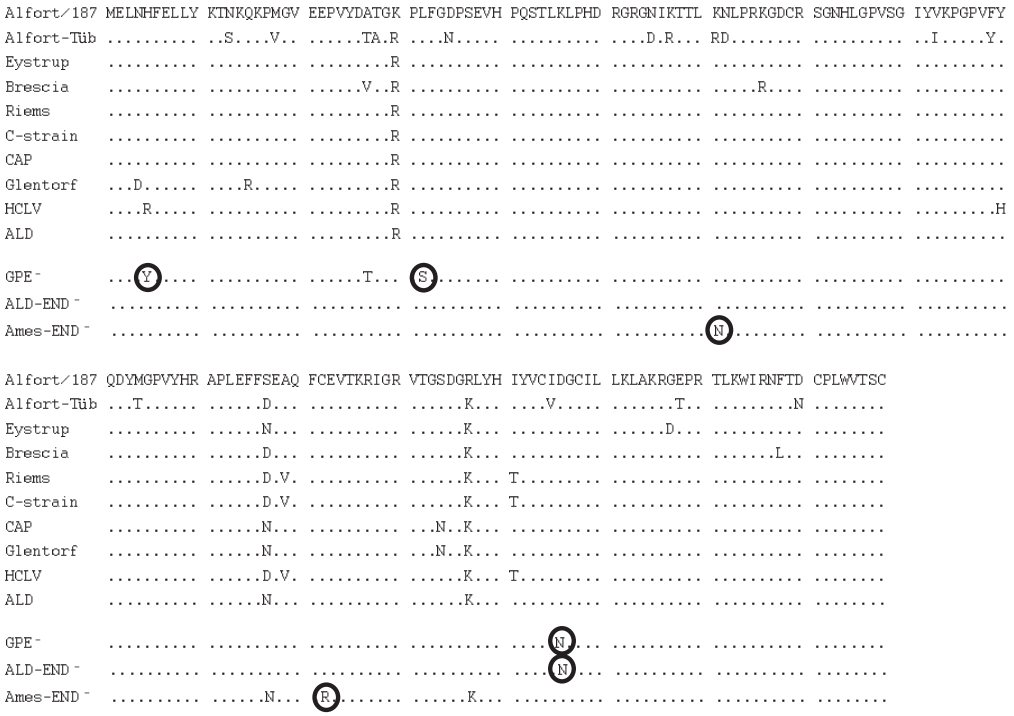

FIG. 3. Alignment of the N^{pro} amino acid sequences of various END-positive and END-negative CSFV strains. Ten END-positive CSFV strains were compared with the END-negative strains GPE^-, ALD-END^-, and Ames-END^-. The respective GenBank accession numbers are as follows: Alfort/187, X87939; Alfort-Tüb, J04358; Eystrup, AF326963; Brescia, AF091661; Riems, AY259122: C-strain, Z46258; CAP, X96550; Glentorf, U45478; HCLV, AF091507; ALD, D49532; and GPE^-, D49533. Sequences for ALD-END^- and Ames-END^- were obtained from Y. Sakoda. The amino acids unique to the END-negative strains are circled.

that partially maintains protease activity can fully abolish the inhibition of the IFN-β promoter activity. These results were confirmed with bicistronic viruses carrying the respective mutations of the proteolytic domains (data not shown). Altogether, based on the results of this study, the autoprotease activity is not required for N^{pro} of CSFV to block IFN-α/β induction.

Alignment of amino acid sequences of N^{pro} of various CSFV strains reveals residues unique to CSFV strains that induce IFN-α/β. The work described above demonstrated that the N^{pro}-mediated block of IFN-α/β induction is independent of an active protease. Consequently, we sought to identify single residues influencing N^{pro} interference with the IFN-α/β induction pathway, independently of the proteolytic function of N^{pro}. For this, we took advantage of the END^- CSFV described to induce IFN-α/β in various cell culture systems. Figure 3 shows an alignment of the N^{pro} amino acid sequences of the END^- strains GPE^-, ALD-END^-, and Ames-END^- and of a selection of END^+ CSFV strains. Special attention was given to the amino acids unique to these END^- strains. The alignment revealed H_5Y, $L_{32}S$, $K_{61}N$, $C_{112}R$, and $D_{136}N$ as candidate mutations that might abolish the N^{pro}-mediated IRF3 degradation and consequently confer the IFN-α/β induction phenotype to CSFV.

Amino acids C_{112} and D_{136} are essential for N^{pro}-mediated degradation of IRF3. To determine whether the residues identified above were critical for the N^{pro}-mediated IRF3 degradation, we constructed CSFV mutants with multiple or single amino acid substitutions in the N^{pro} of the vA187-1 backbone virus (Fig. 4A). Following infection of PK-15 cells with the respective mutant and parent viruses, IRF3 degradation was analyzed and IFN-α/β bioactivity measured. For this work, the original GPE^- strain was amplified on SK-6 and PK-15 cells. Interestingly, the steady-state titer of GPE^- virus recovered from SK-6 cells was approximately 10^2 $TCID_{50}$/ml higher than the titer obtained with PK-15 cells. As expected from its END^- phenotype, the GPE^- virus induced IFN-α/β in PK-15 cells but not in SK-6 cells (data not shown). The latter do not produce IFN-α/β upon stimulation with double-stranded RNA (37). Infection of PK-15 cells with vA187-ΔN^{pro} and with the chimeric virus vA187-N^{pro}(GPE^-) led to IFN-α/β induction, along with a lack of IRF3 degradation; this contrasted with infection by the vA187-1 parent virus (Fig. 4B). These results showed that the N^{pro} gene of GPE^- was sufficient to confer the GPE^- phenotype to vA187-1. We also observed IFN-α/β induction and lack of IRF3 degradation in PK-15 cells following infection with a chimeric vA187-1 virus carrying the N^{pro} gene of the Ames-END^- strain (data not shown).

We then systematically introduced the candidate amino acid substitutions found in the GPE^-, ALD-END^-, and Ames-END^- strains (Fig. 3 and 4A), in combination or as single mutations, into the N^{pro} gene of the vA187-1 backbone (Fig. 4A). These mutant viruses were then analyzed for their capacity to induce IFN-α/β and degrade IRF3 in PK-15 cells. With this approach, we identified two single-amino-acid substitutions, namely, a cysteine-to-arginine substitution at position 112 ($C_{112}R$) and an aspartic acid-to-asparagine substitution at position 136 ($D_{136}N$), that are individually responsible for the

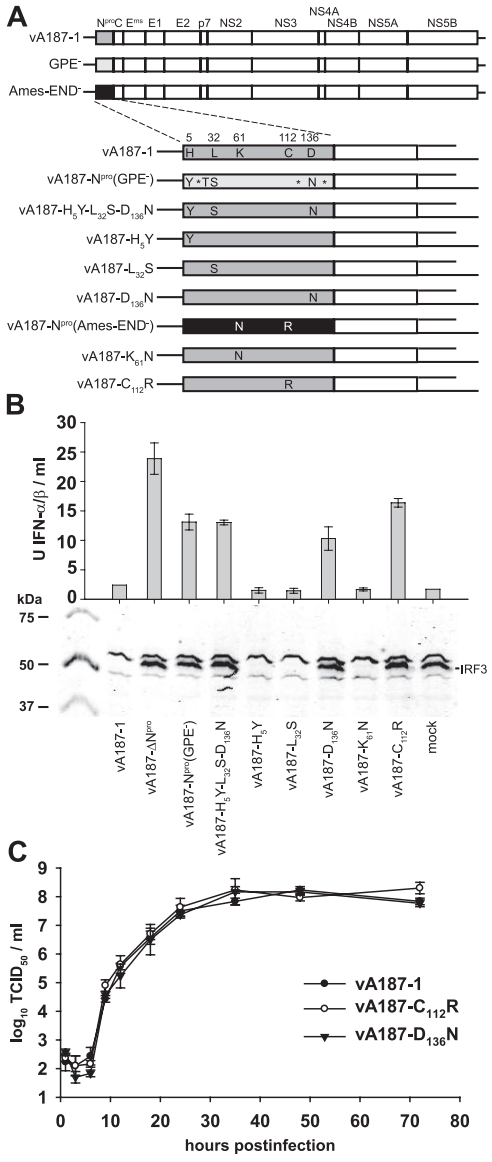

FIG. 4. Amino acids C_{112} and D_{136} are essential for N^{pro}-mediated degradation of IRF3. (A) The nucleotide positions encoding amino acids that are unique to N^{pro} of the CSFV strains GPE^- (light gray) and Ames-END^- (black) and that were introduced in combination or individually in the vA187-1 backbone (dark gray) are represented schematically. For N^{pro} of GPE^-, T is a threonine substitution at position 27 that is not unique to the END^- strains (see Fig. 3), and the asterisks represent silent nucleotide substitutions. (B) PK-15 cells were infected with the mutant viruses as indicated. The IFN-α/β bioactivity was measured in the cell culture supernatant at 65 h postinfection using the Mx/CAT reporter gene assay. Cell extracts were analyzed for IRF3 expression at 48 h postinfection by Western blotting and immunodetection with a rabbit antiserum against porcine IRF3. The position of the IRF3 protein is indicated. (C) SK-6 cells were infected at a multiplicity of infection of 0.1 $TCID_{50}$/cell, and total virus accumulation was determined between 1 and 72 h postinfection as indicated on the *x* axis. Each curve represents the mean from three independent infections, with error bars showing the standard deviations.

phenotypic switch from END^+ to END^- in the three END^- viruses analyzed. Each of these two mutations abolished the capacity of N^{pro} to degrade IRF3 (Fig. 4B) but did not alter the replication characteristics of the virus in SK-6 cells (Fig. 4C).

The cysteine at position 112 and a negative charge at position 136 are required for N^{pro} to block the induction of IFN-β. Having demonstrated that C_{112} and D_{136} are key residues of N^{pro} for mediating IRF3 degradation and thus preventing IFN-β induction, we next analyzed the amino acid requirement at these two positions. Flag-tagged N^{pro} mutants were analyzed in the transient IFN-β promoter reporter assay for their capacity to prevent poly(IC)-mediated IFN-β induction. At position 112 of N^{pro} of the vA187-1 strain, mutation of the cysteine to a basic, acidic, or hydrophobic amino acid abolished the N^{pro} function. A serine at this position partially retained the N^{pro} function. Conversely, replacement of the arginine of N^{pro} of the Ames-END^- strain with a cysteine ($R_{112}C$) restored the capacity of N^{pro} to prevent IFN-β promoter activation (Fig. 5A). This suggests a strict requirement for a cysteine residue at position 112 of N^{pro} for its function in prevention of IFN-β induction. At position 136, the aspartic acid could be replaced with a glutamic acid without any loss of function. All other nonconservative substitutions, in particular the substitution with an arginine, abolished the N^{pro}-mediated inhibition of IFN-β promoter activation; this demonstrated the requirement for a negatively charged amino acid at this position (Fig. 5B). Interestingly, the two substitutions $S_{32}L$ and $N_{136}D$ together were required for conferring to N^{pro} of CSFV GPE^- the capacity to block poly(IC)-mediated induction of the IFN-β promoter (data not shown).

The amino acids C_{112} and D_{136} are essential for the interaction of N^{pro} with IRF3. Negatively charged residues and cysteines are likely to be involved in protein-protein interactions. In a previous report, we have shown that N^{pro} interacts with IRF3 (5). We therefore asked whether the residues C_{112} and D_{136} were involved in this interaction. To this end, we performed a mammalian two-hybrid assay with porcine IRF3 and the $C_{112}R$ and the $D_{136}N$ mutants of N^{pro}. Coexpression of N^{pro} fused to the yeast GAL4 DNA-binding domain and of porcine IRF3 fused to the VP16 activation domain resulted in 300-fold induction of the GAL4 promoter-driven luciferase expression, compared to the empty vectors, confirming the interaction of N^{pro} with IRF3 (Fig. 6). Both the $C_{112}R$ and the $D_{136}N$ mutations completely abolished this interaction, with only a twofold induction. The $C_{69}A$ mutation, which does not alter the N^{pro}-mediated IRF3 degradation (data not shown) and block of IFN-β induction (Fig. 2), does not abrogate the interaction of N^{pro} with IRF3. There were comparable protein expression levels of IRF3 and parent and mutant N^{pro} fusion proteins in parallel cellular extracts (Fig. 6). These data strongly indicate a crucial role of the two residues C_{112} and D_{136} and of the carboxy-terminal region of CSFV N^{pro} in the interaction of N^{pro} with IRF3.

The highly virulent CSFV strain vEy-37, in which the N^{pro}-mediated degradation of IRF3 is specifically knocked out, retains its virulence. Inactivation of a viral gene function that interferes with the IFN system is expected to result in attenuation. Indeed, deletion of the entire N^{pro} gene from the highly virulent vEy-37 strain resulted in an avirulent virus (25). However, a complete gene deletion may affect different functions. In order to specifically investigate the role of the N^{pro}-mediated IRF3 degradation in pathogenesis of CSF, we studied the pathogenicity in pigs of vEy-37-derived viruses carrying minimal mutations in N^{pro} specifically affecting N^{pro}-mediated

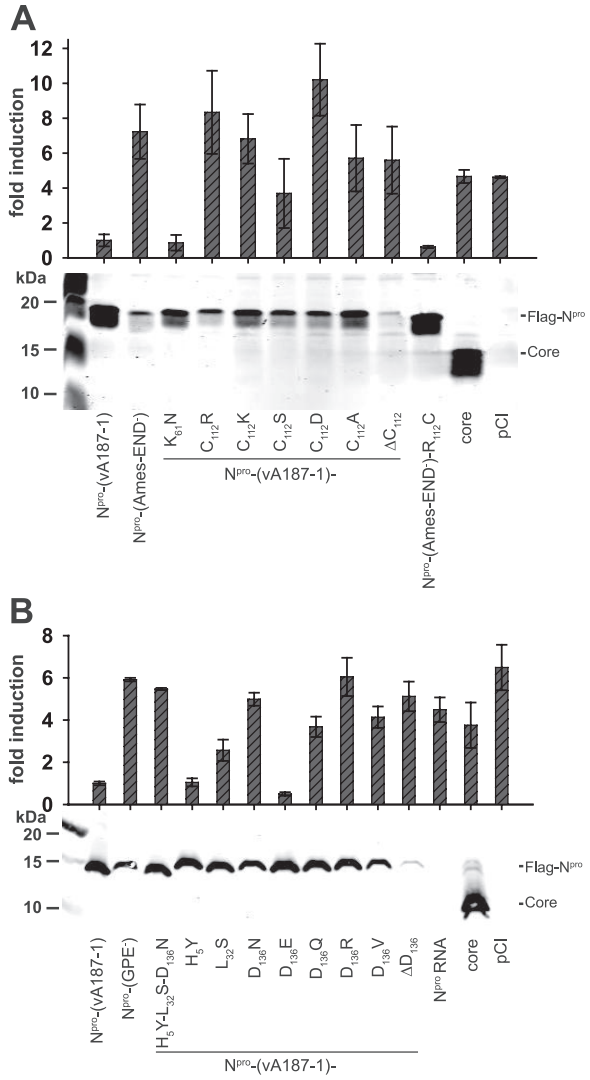

FIG. 5. The cysteine at position 112 and a negative charge at position 136 are required for N^{pro} to block the induction of IFN-β. HEK 293T cells (two replica wells each) were transfected with a mixture of the reporter plasmid p125Luc for IFN-β promoter activity, plasmid phRL-SV40 for internal normalization, and the respective plasmid expressing Flag-tagged wild-type N^{pro} of the virus indicated in brackets or N^{pro} of vA187-1 with amino acid substitutions or deletions (ΔC_{112} or ΔD_{136}) related to either the Ames-END$^-$ (A) or the GPE$^-$ (B) virus strain, as indicated on the *x* axis. The empty vector (pCI) and plasmids encoding the CSFV nucleocapsid protein (core) or nontranslatable N^{pro} RNA served as controls. Cells of one replica well were then stimulated with poly(IC) transfection, and cells of the other well were mock transfected. The firefly luciferase activity was measured and normalized with the constitutively expressed *Renilla* luciferase activity. Each bar represents the relative induction of poly(IC)-treated versus mock-treated cells. The results are the means for three independent sets of transfections, with error bars showing the standard deviations. Total protein was extracted from parallel wells, and Flag-tagged N^{pro} was analyzed by Western blotting using an anti-Flag MAb. The core protein was detected with a rabbit anti-C serum.

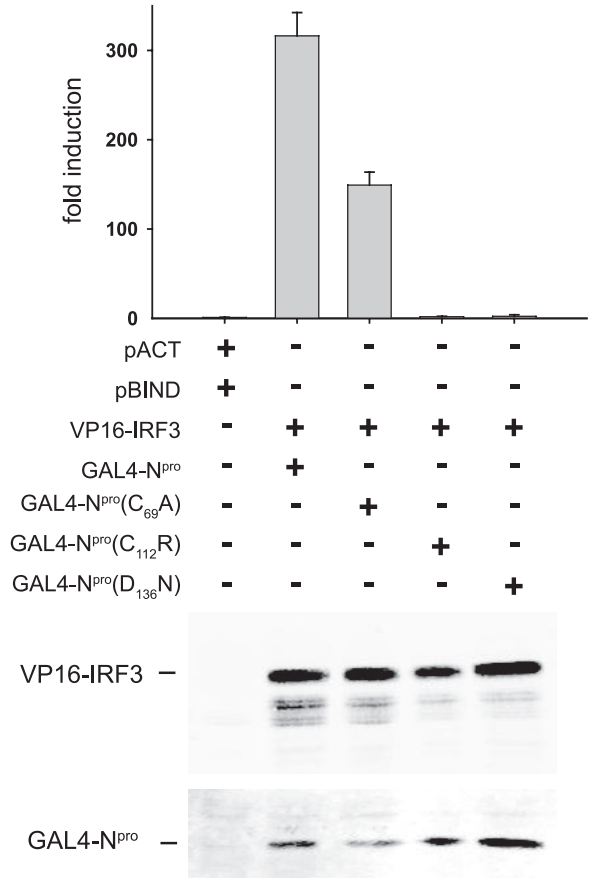

FIG. 6. The mutations $C_{112}R$ and $D_{136}N$ abolish the interaction of N^{pro} with IRF3. HEK 293T cells were transfected with a mixture of the negative control plasmids pACT and pBIND or with a combination of pFN10A(ACT)-IRF3 expressing the porcine IRF3 fused to the VP16 transactivator (VP16-IRF3) and of pFN11A(BIND)-derived plasmids expressing a GAL4-N^{pro} fusion protein or N^{pro} mutants thereof, as indicated. After 24 h of incubation, the firefly luciferase activity was measured and normalized with the *Renilla* luciferase activity constitutively expressed from the pFN11A(BIND)-derived plasmids. The relative firefly luciferase induction (fold induction) was calculated as described in Materials and Methods. The bars represent the means of triplicates from a representative experiment, with error bars showing the standard deviations. The expression of VP16-IRF3 and GAL4-N^{pro} from parallel wells of the same experiment was analyzed by Western blotting and immunodetection with the rabbit antisera against porcine IRF3 and N^{pro}, respectively.

IRF3 degradation. First, we introduced the three GPE$^-$-specific substitutions H_5Y, $L_{32}S$, and $D_{136}N$ or the $D_{136}N$ mutation alone in the vEy-37 backbone, resulting in vEy-N^{pro}(GPE$^-$) and vEy-$D_{136}N$, respectively. In order to exclude the accidental introduction of any attenuating mutation elsewhere in the genome during cloning, we reconstructed the parent vEy-37 virus by introducing the wild-type N^{pro} sequence in the vEy-$D_{136}N$ backbone. As expected, vEy-N^{pro}(GPE$^-$) and vEy-$D_{136}N$ induced IFN-α/β in PK-15 cells and did not induce IRF3 degradation, contrasting with the parent vEy-37 (data not shown).

Groups of four 8-week-old SPF pigs were infected via the oronasal route with 10^5 $TCID_{50}$/pig of mutant or parent virus. Infection with vEy-37 resulted in the typical clinical picture of acute CSF with severe symptoms and a biphasic body temperature curve (Fig. 7A and B, right). The pigs were euthanized when the clinical score was between 18 and 20. Surprisingly, pigs infected with the mutant viruses developed severe disease, with a slight delay of 1 day compared to wild-type virus infec-

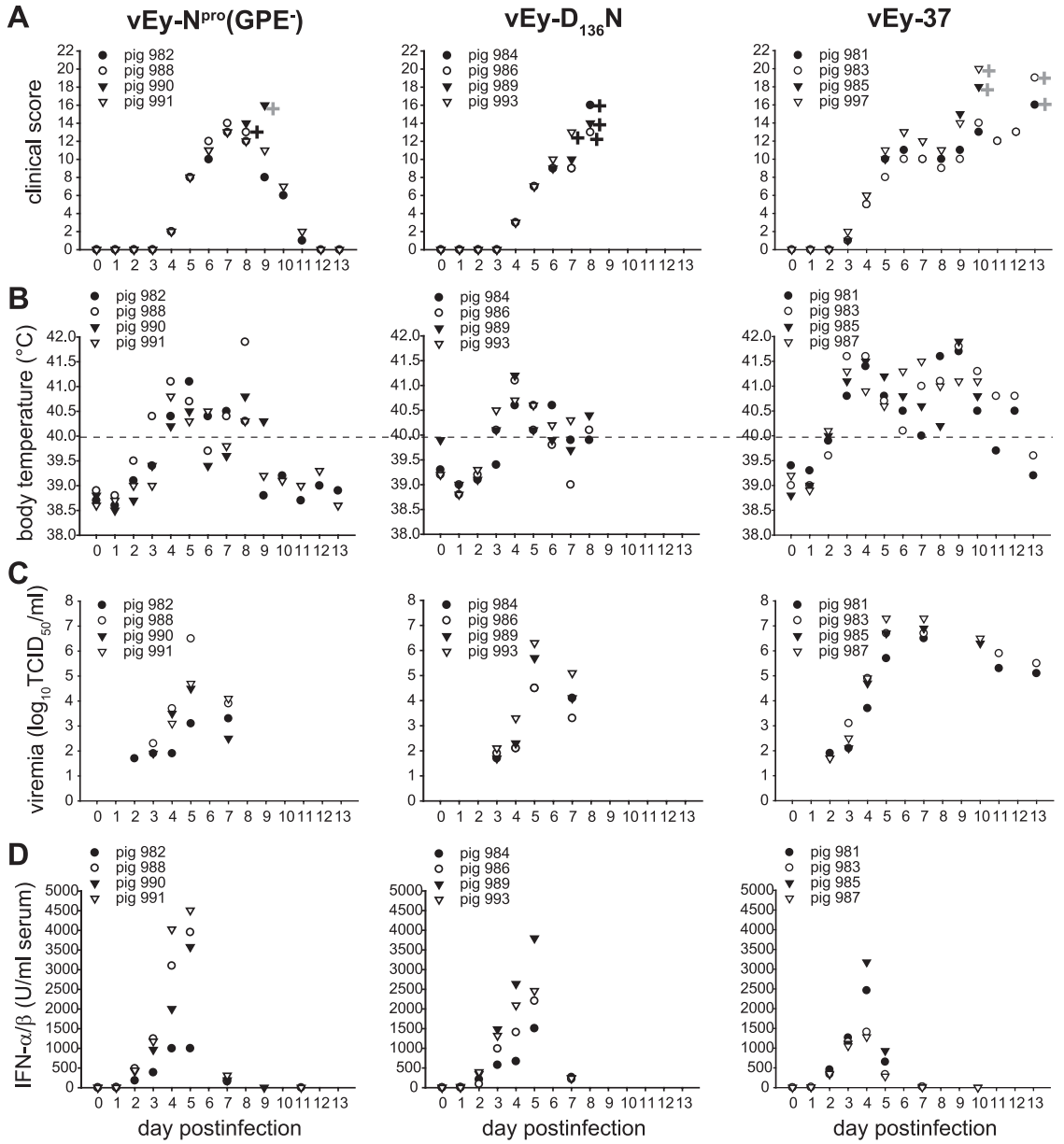

FIG. 7. The viruses vEy-N^{pro}(GPE$^-$) and vEy-D$_{136}$N retain the high virulence of the parent vEy-37 strain. Three groups of four 8-week-old SPF pigs were infected oronasally with 10^5 TCID$_{50}$ of mutant vEy-N^{pro}(GPE$^-$) virus, mutant vEy-D$_{136}$N virus, or the parent vEy-37 virus. Clinical scores (A), body temperatures (B), serum virus titers (C), and serum IFN-α/β levels (D) were monitored daily. A body temperature of above 40.0°C was considered fever. In panels A, the animals that died unexpectedly are marked with a black cross, and the animals that were euthanized are represented with a gray cross.

tion before the onset of clinical signs. One animal infected with vEy-N^{pro}(GPE$^-$) (pig 988), as well as the four animals infected with vEy-D$_{136}$N, died unexpectedly between days 7 and 8 postinfection, although their clinical status (clinical scores between 13 and 16) was not critical (Fig. 7A and B, left and middle). Necropsy of the latter animals, as well as of pig 990 [vEy-N^{pro}(GPE$^-$) group] (Fig. 7, left) euthanized on day 9, revealed marked hemoabdomen and extensive ecchymotic and petechial hemorrhages within various organs, including tonsils, stomach, kidneys, urinary bladder, intestine, and muscles. Two pigs infected with vEy-N^{pro}(GPE$^-$) spontaneously recovered and were free of any symptoms by day 12 postinfection. Sequence analysis of the N^{pro} gene of the virus rescued from organs of animals that had died showed that the mutations had not reverted in any of the pigs (data not shown). Early after infection, similar levels of viremia were observed in the three groups (Fig. 7C). Interestingly, the highest values of IFN-α/β bioactivity were measured in the sera of the animals infected with the N^{pro} mutant viruses (Fig. 7D).

In order to confirm these unexpected observations and to determine whether the exacerbation of disease was associated specifically with the presence of the D$_{136}$N mutation, we re-

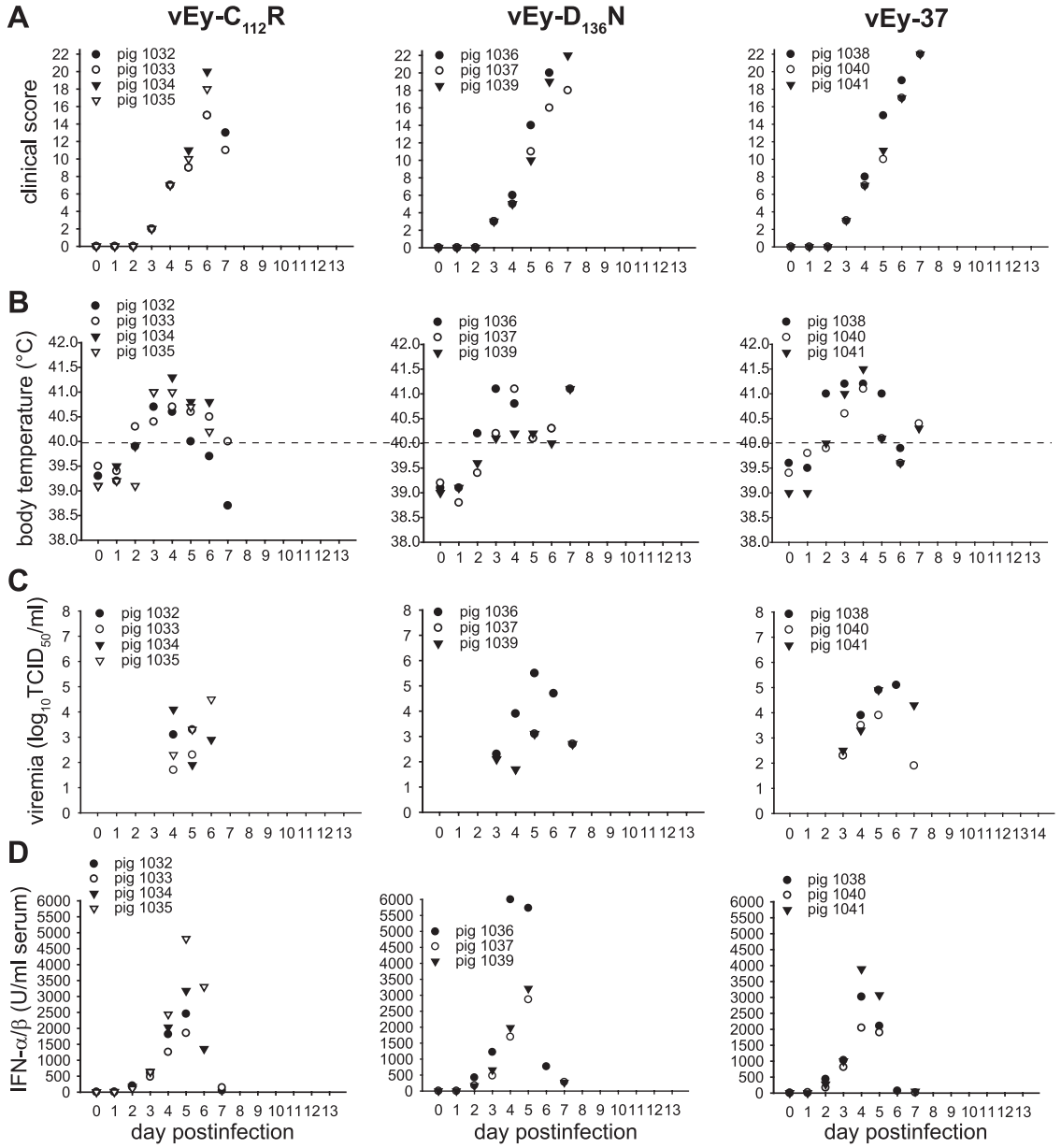

FIG. 8. The mutations $C_{112}R$ and $D_{136}N$, which independently knock out the N^{pro}-mediated degradation of IRF3, do not attenuate the highly virulent vEy-37 strain. Three groups of three or four 10-week-old SPF pigs were infected oronasally with 10^5 $TCID_{50}$ of mutant vEy-$C_{112}R$ virus, mutant vEy-$D_{136}N$ virus, and the parent vEy-37 virus, respectively. Clinical scores (A), body temperatures (B), serum virus titers (C), and serum IFN-α/β levels (D) were monitored daily. A body temperature of above 40.0°C was considered fever. All animals were euthanized at day 6 or 7 after infection, which corresponds to the last dot for each animal.

peated the experiment with the inclusion of a vEy-37-derived mutant carrying the $C_{112}R$ substitution (vEy-$C_{112}R$) instead of the vEy-N^{pro}(GPE^-) virus. The pigs were infected at 10 weeks of age with the same dose of virus as in the previous experiment. Again, only a very little difference was observed between the three groups in terms of clinical score, body temperature, viremia, and IFN-α/β bioactivity in the serum (Fig. 8). The overall picture was reminiscent of the first experiment (compare Fig. 7 and 8). Two of the four pigs infected with vEy-$C_{112}R$ reached clinical scores of 18 and 20 at day 6 postinfection and were euthanized (Fig. 8A, left). All the remaining animals showed severe symptoms 1 day later and were euthanized for animal welfare reasons. Based on the clinical score and body temperature, it is likely that two of the pigs infected with vEy-$C_{112}R$ may have survived the infection (Fig. 8A and B, left). It is noteworthy that the highest IFN-α/β bioactivity was again measured in the sera of animals infected with the N^{pro} mutant viruses, with values between 5,000 and 6,000 U/ml serum (Fig. 8D, left and middle). Taken together, the data from the two experiments demonstrate that the N^{pro}-mediated degradation of IRF3 is not a prerequisite for a highly virulent CSFV to express its virulence.

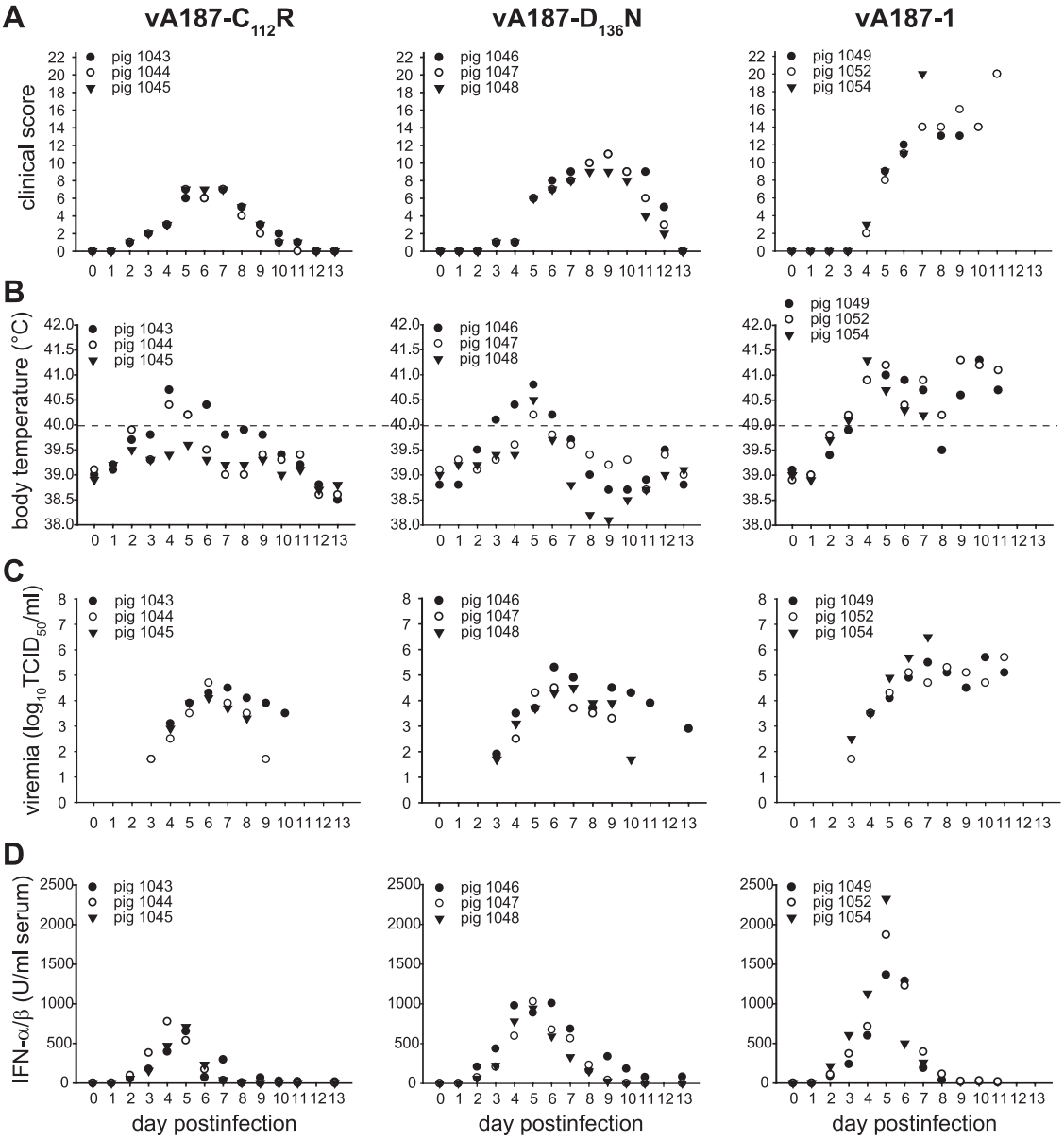

FIG. 9. The mutations $C_{112}R$ and $D_{136}N$ partially attenuate the moderately virulent vA187-1 strain. Three groups of three 9-week-old SPF pigs were infected oronasally with 10^6 $TCID_{50}$ of mutant vA187-$C_{112}R$ virus, mutant vA187-$D_{136}N$ virus, or the parent vA187-1 virus. Clinical scores (A), body temperatures (B), serum virus titers (C), and serum IFN-α/β levels (D) were monitored daily. A body temperature of above 40.0°C was considered fever. All animals were euthanized, either for animal welfare reasons because of severe symptoms or at the end of the experiment, which corresponds to the last dot for each animal.

The moderately virulent CSFV strain vA187-1, in which the N^{pro}-mediated degradation of IRF3 is specifically knocked out, is partially attenuated. The unexpected results with vEy-37-derived N^{pro} mutants in vivo suggest that a highly virulent CSFV does not rely on counteracting the IFN system to replicate in vivo and cause severe disease. In order to better understand the role of N^{pro} in vivo, we sought to verify the findings with a less virulent virus. Accordingly, a third experiment with $C_{112}R$ and $D_{136}N$ mutants of the moderately virulent vA187-1 virus was performed. In the SK-6 cell line, which is incapable of IFN-α/β production, the vA187-$C_{112}R$, the vA187-$D_{136}N$, and the vA187-1 viruses were indistinguishable with respect to their virus replication characteristics, despite the absence of IRF3 degradation with the mutant viruses (Fig. 4B and C). Three groups of three 9-week-old SPF pigs were infected with 10^6 $TCID_{50}$/pig of the respective virus via the oronasal route. Figure 9 shows clinical scores, body temperatures, viremia, and serum IFN-α/β levels monitored as in the previous two experiments. Between days 7 and 11 postinfection, the vA187-1-infected pigs were euthanized when they reached a clinical score of above 18 (Fig. 9A, right). The pigs infected with the vA187-$C_{112}R$ and the vA187-$D_{136}N$ mutant viruses reached maximal clinical scores of 7 and 11, respectively, and completely recovered within 12 to 13 days (Fig. 9A,

left and middle). The body temperatures reflected the milder symptoms observed with the mutant viruses (Fig. 9B). All pigs infected with the mutant viruses could eventually eliminate the virus, whereas the vA187-1-infected animals had virus titers of up to 10^5 $TCID_{50}$/ml serum at the time of euthanasia (Fig. 9C). Interestingly, the overall IFN-α/β levels in the serum were approximately four to five times lower with all three viruses than the corresponding IFN-α/β bioactivities measured with the vEy-37-derived viruses (Fig. 9D and 8D [note the different scales of the *y* axes]); the highest values in the third experiment were observed with vA187-1 virus (Fig. 9D, right). This set of data indicates that the effect of the loss of N^{pro}-mediated IRF3 degradation becomes more apparent with a less virulent virus but results in only a moderate attenuation.

DISCUSSION

N^{pro} of pestiviruses has at least two functions: an autoprotease activity for cotranslational cleavage from the nascent polyprotein and an antagonistic effect on the IFN-α/β induction pathway by targeting IRF3 for proteasomal degradation. All reports of in vivo experiments with pestiviruses in which nearly the entire N^{pro} gene was deleted show that the lack of N^{pro} results in considerable or total attenuation (25, 27, 49). It was therefore plausible to hypothesize that the degradation of IRF3 allows pestiviruses to colonize their host efficiently and express their pathogenic potential. In order to verify this hypothesis, we searched for minimal amino acid substitutions that would specifically abrogate the N^{pro}-mediated block of IFN-α/β induction, to elaborate on the role of this N^{pro} function in vivo.

The N^{pro} autoprotease was identified as a novel type of cysteine protease with some similarity to subtilisin-like proteases (38, 47, 52). Site-directed mutagenesis and translation in cell-free lysates revealed that amino acid residues E_{22}, H_{49}, and C_{69} of N^{pro} were essential for proteolysis (38). Our observation that the deletion of 22 or more amino acids at the amino terminus inactivates the N^{pro} protease is in agreement with these previous studies. Interestingly, the $E_{22}V$ and $C_{69}S$ mutations only partially reduced the processing activity of N^{pro}. This is in disagreement with previously published data showing complete inactivation of the protease with these two substitutions in N^{pro} of BVDV (9, 17) and CSFV (38). This discrepancy may be due in part to the use of rabbit reticulocyte lysate for expression in certain studies (17, 38).

With respect to the control of IFN-α/β induction by CSFV, it was not clear if the protease activity of N^{pro} was involved. An initial study with BVDV suggested that the proteolytic activity of N^{pro} would be required for suppressing IRF3-mediated responses (19). However, subsequent observations showed that the proteasome inhibitors MG132 and epoxomicin prevented IRF3 degradation in the presence of N^{pro} expression by both BVDV and CSFV (5, 9, 17, 45). This would indicate that the loss of IRF3 was more likely due to proteasomal rather than N^{pro}-mediated proteolysis. With N^{pro} harboring mutations that completely inactivate its proteolytic activity, it became clear for both BVDV (9, 14, 17) and CSFV (this study) that N^{pro} counteracts IFN-α/β production independently of an active protease. This is particularly obvious in the present study, where we monitored N^{pro}-mediated protein processing and IFN-β promoter induction using the same expression constructs in parallel transfections. Mutation of glutamic acid at position 22 to valine ($E_{22}V$) both reduces the proteolytic activity of N^{pro} and abolishes the capacity of N^{pro} to block IFN-β induction. This suggests that the autoprotease domain and the IRF3-degrading domain structurally overlap, sharing common essential residues. It was also proposed that the BVDV N^{pro}-mediated block of IFN-α/β induction involved the amino-terminal (14) and carboxy-terminal (9, 17) regions of N^{pro}. For CSFV, we have now shown that the amino terminus is dispensable for blocking IFN-α/β induction; N^{pro} functions in the absence of the 19 amino-terminal amino acids, both in a viral context and in transient assays.

In order to pursue our search for amino acid residues of N^{pro} that are essential for N^{pro}-mediated degradation of IRF3 and inhibition of IFN-α/β induction, we focused on amino acids unique to END^- CSFV strains. The END^- strain GPE^- has been used as a live attenuated vaccine in Japan. It was derived from the virulent strain ALD by serial passage in guinea pig kidney cells. During this procedure, the virus was attenuated, additionally losing its capacity to prevent IFN-α/β induction. The same phenomenon was observed with the END^- CSFV strains Ames-END^- and ALD-END^- (39, 41). All of these END^- viruses have a functional autoprotease, which led us to hypothesize that residues of N^{pro} found solely in these strains might be critical for the autoprotease-independent function of N^{pro} in the IFN induction pathway. After introduction of these individual changes in a standard CSFV backbone, we did indeed identify two single amino acids critical for N^{pro}-mediated IRF3 degradation: C_{112} and D_{136}.

We proposed a model for CSF pathogenesis in which CSFV counteracts IFN-α/β induction by depleting IRF3 in the primary target cells, allowing the virus to establish a productive infection. In a second stage, the virus infects plasmacytoid dendritic cells that can produce IFN-α independently of IRF3 (18); this results in overproduction of IFN-α, orchestrating the immunopathological effects typical of CSF (4, 5, 48). The in vivo data in the present report with CSFV mutants specifically lacking the capacity to degrade IRF3 demonstrate that the situation is clearly more complicated. In particular, the pertinence of N^{pro}-mediated IRF3 degradation to disease development during the acute phase of CSF is limited. The loss of N^{pro}-mediated IRF3 degradation did not result in attenuation of highly virulent CSFV, while only a moderate attenuation was observed with a less virulent strain. Certainly, it is important to note that all experiments were performed with 8- to 10-week-old pigs, and we cannot discount the possibility that N^{pro}-mediated attenuation might be more prominent in older animals. Nevertheless, in contrast to our original hypothesis based on observations with other virus systems, our data now indicate that N^{pro}-mediated IRF3 degradation has a minor contribution in terms of permitting virus replication and disease development during the acute phase of CSF. We therefore speculate that N^{pro} plays a more important role in the sense of promoting a longer duration of virus infection in its target cells and in the host. This might be important also for virus persistence in the wild boar, the natural CSFV reservoir. In domestic pigs, the IRF3-degrading function of N^{pro} would certainly play a role with moderately virulent or low-virulent strains that tend to induce more chronic disease. Chronic

CSFV infections can last up to 100 days (51). N^{pro} might allow the virus to persist in a particular cell type or organ, for example, in vascular endothelial cells (6), lymph nodes, spleen, and intestine, in particular the gut-associated lymphoid tissue (10, 42). It is also possible that N^{pro} is important for establishing persistent infections in offspring of animals infected during pregnancy. While the importance of this pathway of infection is not clear for CSF, it represents a key characteristic of BVDV infections in cattle. Persistence of noncytopathogenic BVDV in the bovine fetus was associated with failure of the virus to induce IFN-α/β (8). A recent report suggests that N^{pro} and E^{rns} of BVDV are involved in persistence after transplacental infection (27). Such studies with the present mutants generated for BVDV are required to clarify this point, in particular whether both the RNase function of E^{rns} and the IRF3 degradation mediated by N^{pro} are required for the establishment of persistent infections.

Both the present work and previous studies (48) have noted an early, strong, systemic IFN-α response following CSFV infection. The fact that the viral titers continue to rise despite this response indicates that the contribution of the IFN-α/β system in limiting virus replication during the acute phase of CSF is incomplete. This also suggests that CSFV possesses an inherent resistance to the antiviral effect of IFN-α/β, which is independent of the function of N^{pro} described in the present work. Our observations relate to the data of Schweizer and coworkers showing resistance of the pestivirus BVDV to IFN-α/β in vitro once the infection is established, while the virus does not apparently target the IFN-α/β signaling pathway (43).

The disease exacerbation observed with the vEy-37 N^{pro} mutant viruses, which induced particularly high levels of IFN-α/β, is interesting with respect to the previously proposed role of aberrant IFN-α/β responses in CSF immunopathogenesis (48). This also supports the idea that IFN-α/β responses contribute more to disease development than to protection with virulent strains of CSFV. However, additional factors such as proinflammatory cytokines may well contribute to CSF pathogenesis (31), because the highest levels of IFN-α did not necessarily correlate with the most severe clinical scores or death.

Taken together, the results of the present study show that specific abrogation of a viral function that efficiently prevents the induction of IFN-α/β in non-plasmacytoid dendritic cells does not necessarily result in attenuation. These data shed new light not only on the role of the IFN-α/β system during the early phase of CSFV infection in vivo but also on the role of N^{pro} in the biology of pestiviruses.

ACKNOWLEDGMENTS

This work was supported in part by Swiss National Science Foundation grants 3100A0-102066 and 3100A0-116608 and in part by Swiss Federal Veterinary Office grant 1.03.04. C.G.L. was supported by a Students Training in Advanced Research summer fellowship, sponsored by NIH/NCRR, to the School of Veterinary Medicine, University of California, Davis, CA.

We thank C. Griot for continuous support and Kenneth C. McCullough for critical reading of the manuscript. We also thank Alexandra Meindl, Sandra Blome, and Irene Greiser-Wilke (Community Reference Laboratory for CSF, School of Veterinary Medicine, Hannover, Germany) for the GPE^- strain, Martin D. Fray (Institute of Animal Health, Compton, Newbury, Berkshire, United Kingdom) for the Mx/CAT reporter gene assay, and Takashi Fujita (Kyoto University, Japan) for the reporter plasmid p125Luc.

REFERENCES

1. **Baigent, S. J., S. Goodbourn, and J. W. McCauley.** 2004. Differential activation of interferon regulatory factors-3 and -7 by non-cytopathogenic and cytopathogenic bovine viral diarrhoea virus. Vet. Immunol. Immunopathol. **100:**135–144.
2. **Baigent, S. J., G. Zhang, M. D. Fray, H. Flick-Smith, S. Goodbourn, and J. W. McCauley.** 2002. Inhibition of beta interferon transcription by noncytopathogenic bovine viral diarrhea virus is through an interferon regulatory factor 3-dependent mechanism. J. Virol. **76:**8979–8988.
3. **Balmelli, C., N. Ruggli, K. C. McCullough, and A. Summerfield.** 2005. Fibrocytes are potent stimulators of anti-virus cytotoxic T cells. J. Leukoc. Biol. **77:**923–933.
4. **Balmelli, C., I. E. Vincent, H. Rau, L. Guzylack-Piriou, K. C. McCullough, and A. Summerfield.** 2005. FcgammaRII-dependent sensitisation of natural interferon-producing cells for viral infection and interferon-alpha responses. Eur. J. Immunol. **35:**2406–2415.
5. **Bauhofer, O., A. Summerfield, Y. Sakoda, J. D. Tratschin, M. A. Hofmann, and N. Ruggli.** 2007. N^{pro} of classical swine fever virus interacts with interferon regulatory factor 3 and induces its proteasomal degradation. J. Virol. **81:**3087–3096.
6. **Bensaude, E., J. L. E. Turner, P. R. Wakeley, D. A. Sweetman, C. Pardieu, T. W. Drew, T. Wileman, and P. P. Powel.** 2004. Classical swine fever virus induces proinflammatory cytokines and tissue factor expression and inhibits apoptosis and interferon synthesis during the establishment of long-term infection of porcine vascular endothelial cells. J. Gen. Virol. **85:**1029–1037.
7. **Carrasco, C. P., R. C. Rigden, I. E. Vincent, C. Balmelli, M. Ceppi, O. Bauhofer, V. Tache, B. Hjertner, F. McNeilly, H. G. van Gennip, K. C. McCullough, and A. Summerfield.** 2004. Interaction of classical swine fever virus with dendritic cells. J. Gen. Virol. **85:**1633–1641.
8. **Charleston, B., M. D. Fray, S. Baigent, B. V. Carr, and W. I. Morrison.** 2001. Establishment of persistent infection with non-cytopathic bovine viral diarrhoea virus in cattle is associated with a failure to induce type 1 interferon. J. Gen. Virol. **82:**1893–1897.
9. **Chen, Z., R. Rijnbrand, R. K. Jangra, S. G. Devaraj, L. Qu, Y. Ma, S. M. Lemon, and K. Li.** 2007. Ubiquitination and proteasomal degradation of interferon regulatory factor-3 induced by N^{pro} from a cytopathic bovine viral diarrhea virus. Virology **366:**277–292.
10. **Choi, C., and C. Chae.** 2003. Localization of classical swine fever virus from chronically infected pigs by in situ hybridization and immunohistochemistry. Vet. Pathol. **40:**107–113.
11. **Fauquet, C. M., M. A. Mayo, J. Maniloff, U. Desselberger, and L. A. Ball.** 2005. Virus taxonomy: classification and nomenclature of viruses. VIIIth report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, United Kingdom.
12. **Fray, M. D., G. E. Mann, and B. Charleston.** 2001. Validation of an Mx/CAT reporter gene assay for the quantification of bovine type-I interferon. J. Immunol. Methods **249:**235–244.
13. **Garcia-Sastre, A., and C. A. Biron.** 2006. Type 1 interferons and the virus-host relationship: a lesson in detente. Science **312:**879–882.
14. **Gil, L. H., I. H. Ansari, V. Vassilev, D. Liang, V. C. Lai, W. Zhong, Z. Hong, E. J. Dubovi, and R. O. Donis.** 2006. The amino-terminal domain of bovine viral diarrhea virus N^{pro} protein is necessary for alpha/beta interferon antagonism. J. Virol. **80:**900–911.
15. **Greiser-Wilke, I., V. Moennig, C. O. Coulibaly, J. Dahle, L. Leder, and B. Liess.** 1990. Identification of conserved epitopes on a hog cholera virus protein. Arch. Virol. **111:**213–225.
16. **Haller, O., and F. Weber.** 2007. Pathogenic viruses: smart manipulators of the interferon system. Curr. Top. Microbiol. Immunol. **316:**315–334.
17. **Hilton, L., K. Moganeradj, G. Zhang, Y. H. Chen, R. E. Randall, J. W. McCauley, and S. Goodbourn.** 2006. The N^{pro} product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and targets it for proteasomal degradation. J. Virol. **80:**11723–11732.
18. **Honda, K., H. Yanai, H. Negishi, M. Asagiri, M. Sato, T. Mizutani, N. Shimada, Y. Ohba, A. Takaoka, N. Yoshida, and T. Taniguchi.** 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature **434:**772–777.
19. **Horscroft, N., D. Bellows, I. Ansari, V. C. Lai, S. Dempsey, D. Liang, R. Donis, W. Zhong, and Z. Hong.** 2005. Establishment of a subgenomic replicon for bovine viral diarrhea virus in Huh-7 cells and modulation of interferon-regulated factor 3-mediated antiviral response. J. Virol. **79:**2788–2796.
20. **Inaba, Y., T. Omori, and T. Kumagai.** 1963. Detection and measurement of non-cytopathogenic strains of virus diarrhea of cattle by the END method. Arch. Gesamte Virusforsch. **13:**425–429.
21. **Kumagai, T., T. Shimizu, and M. Matumoto.** 1958. Detection of hog cholera virus by its effect on Newcastle disease virus in swine tissue culture. Science **128:**366.
22. **La Rocca, S. A., R. J. Herbert, H. Crooke, T. W. Drew, T. E. Wileman, and P. P. Powell.** 2005. Loss of interferon regulatory factor 3 in cells infected with classical swine fever virus involves the N-terminal protease, N^{pro}. J. Virol. **79:**7239–7247.
23. **Lindenbach, B. D., H. J. Thiel, and C. M. Rice.** 2007. Flaviviridae: the

viruses and their replication, p. 1101–1152. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 5th ed., vol 1. Lippincott-Raven Publishers, Philadelphia, PA.

24. **Maurer, R., P. Stettler, N. Ruggli, M. A. Hofmann, and J. D. Tratschin.** 2005. Oronasal vaccination with classical swine fever virus (CSFV) replicon particles with either partial or complete deletion of the E2 gene induces partial protection against lethal challenge with highly virulent CSFV. Vaccine **23:** 3318–3328.
25. **Mayer, D., M. A. Hofmann, and J. D. Tratschin.** 2004. Attenuation of classical swine fever virus by deletion of the viral N^{pro} gene. Vaccine **22:**317–328.
26. **Mayer, D., T. M. Thayer, M. A. Hofmann, and J. D. Tratschin.** 2003. Establishment and characterisation of two cDNA-derived strains of classical swine fever virus, one highly virulent and one avirulent. Virus Res. **98:**105–116.
27. **Meyers, G., A. Ege, C. Fetzer, F. M. von, K. Elbers, V. Carr, H. Prentice, B. Charleston, and E. M. Schurmann.** 2007. Bovine viral diarrhea virus: prevention of persistent fetal infection by a combination of two mutations affecting E^{rns} RNase and N^{pro} protease. J. Virol. **81:**3327–3338.
28. **Mittelholzer, C., C. Moser, J. D. Tratschin, and M. A. Hofmann.** 2000. Analysis of classical swine fever virus replication kinetics allows differentiation of highly virulent from avirulent strains. Vet. Microbiol. **74:**293–308.
29. **Mittelholzer, C., C. Moser, J. D. Tratschin, and M. A. Hofmann.** 1997. Generation of cytopathogenic subgenomic RNA of classical swine fever virus in persistently infected porcine cell lines. Virus Res. **51:**125–137.
30. **Moser, C., P. Stettler, J. D. Tratschin, and M. A. Hofmann.** 1999. Cytopathogenic and noncytopathogenic RNA replicons of classical swine fever virus. J. Virol. **73:**7787–7794.
31. **Nunez, A., J. C. GomezVillamandos, P. J. SanchezCordon, M. F. deMarco, M. Pedrera, F. J. Salguero, and L. Carrasco.** 2005. Expression of proinflammatory cytokines by hepatic macrophages in acute classical swine fever. J. Comp. Pathol. **133:**23–32.
32. **Paton, D. J., and I. GreiserWilke.** 2003. Classical swine fever—an update. Res. Vet. Sci. **75:**169–178.
33. **Peterhans, E., T. W. Jungi, and M. Schweizer.** 2006. How the bovine viral diarrhea virus outwits the immune system. Dtsch. Tierarztl. Wochenschr. **113:**124–129.
34. **Randall, R. E., and S. Goodbourn.** 2008. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. J. Gen. Virol. **89:**1–47.
35. **Ruggli, N., B. H. Bird, L. Liu, O. Bauhofer, J. D. Tratschin, and M. A. Hofmann.** 2005. N^{pro} of classical swine fever virus is an antagonist of double-stranded RNA-mediated apoptosis and IFN-alpha/beta induction. Virology **340:**265–276.
36. **Ruggli, N., J. D. Tratschin, C. Mittelholzer, and M. A. Hofmann.** 1996. Nucleotide sequence of classical swine fever virus strain Alfort/187 and transcription of infectious RNA from stably cloned full-length cDNA. J. Virol. **70:**3478–3487.
37. **Ruggli, N., J. D. Tratschin, M. Schweizer, K. C. McCullough, M. A. Hofmann, and A. Summerfield.** 2003. Classical swine fever virus interferes with cellular antiviral defense: evidence for a novel function of N^{pro}. J. Virol. **77:**7645–7654.
38. **Rümenapf, T., R. Stark, M. Heimann, and H. J. Thiel.** 1998. N-terminal protease of pestiviruses: identification of putative catalytic residues by site-directed mutagenesis. J. Virol. **72:**2544–2547.
39. **Sakoda, Y., M. Hikawa, T. Tamura, and A. Fukusho.** 1998. Establishment of a serum-free culture cell line, CPK-NS, which is useful for assays of classical swine fever virus. J. Virol. Methods **75:**59–68.
40. **Sakoda, Y., S. Ozawa, S. Damrongwatanapokin, M. Sato, K. Ishikawa, and A. Fukusho.** 1999. Genetic heterogeneity of porcine and ruminant pestiviruses mainly isolated in Japan. Vet. Microbiol. **65:**75–86.
41. **Sakoda, Y., O. Yamaguchi, and A. Fukusho.** 1998. A new assay for classical swine fever virus based on cytopathogenicity in porcine kidney cell line FS-L3. J. Virol. Methods **70:**93–101.
42. **Sanchez-Cordon, P. J., S. Romanini, F. J. Salguero, E. Ruiz-Villamor, L. Carrasco, and J. C. Gomez-Villamandos.** 2003. A histopathologic, immunohistochemical, and ultrastructural study of the intestine in pigs inoculated with classical swine fever virus. Vet. Pathol. **40:**254–262.
43. **Schweizer, M., P. Matzener, G. Pfaffen, H. Stalder, and E. Peterhans.** 2006. "Self" and "nonself" manipulation of interferon defense during persistent infection: bovine viral diarrhea virus resists alpha/beta interferon without blocking antiviral activity against unrelated viruses replicating in its host cells. J. Virol. **80:**6926–6935.
44. **Schweizer, M., and E. Peterhans.** 2001. Noncytopathic bovine viral diarrhea virus inhibits double-stranded RNA-induced apoptosis and interferon synthesis. J. Virol. **75:**4692–4698.
45. **Seago, J., L. Hilton, E. Reid, V. Doceul, J. Jeyatheesan, K. Moganeradj, J. McCauley, B. Charleston, and S. Goodbourn.** 2007. The N^{pro} product of classical swine fever virus and bovine viral diarrhea virus uses a conserved mechanism to target interferon regulatory factor-3. J. Gen. Virol. **88:**3002–3006.
46. **Shimizu, Y., S. Furuuchi, T. Kumagai, and J. Sasahara.** 1970. A mutant of hog cholera virus inducing interference in swine testicle cell cultures. Am. J. Vet. Res. **31:**1787–1794.
47. **Stark, R., G. Meyers, T. Rümenapf, and H. J. Thiel.** 1993. Processing of pestivirus polyprotein: cleavage site between autoprotease and nucleocapsid protein of classical swine fever virus. J. Virol. **67:**7088–7095.
48. **Summerfield, A., M. Alves, N. Ruggli, M. G. de Bruin, and K. C. McCullough.** 2006. High IFN-alpha responses associated with depletion of lymphocytes and natural IFN-producing cells during classical swine fever. J. Interferon Cytokine Res. **26:**248–255.
49. **Tratschin, J. D., C. Moser, N. Ruggli, and M. A. Hofmann.** 1998. Classical swine fever virus leader proteinase N^{pro} is not required for viral replication in cell culture. J. Virol. **72:**7681–7684.
50. **Van Cleve, W., E. Amaro-Carambot, S. R. Surman, J. Bekisz, P. L. Collins, K. C. Zoon, B. R. Murphy, M. H. Skiadopoulos, and E. J. Bartlett.** 2006. Attenuating mutations in the P/C gene of human parainfluenza virus type 1 (HPIV1) vaccine candidates abrogate the inhibition of both induction and signaling of type I interferon (IFN) by wild-type HPIV1. Virology **352:**61–73.
51. **van Oirschot, J. T.** 1988. Description of the virus infection, p. 1–25. *In* B. Liess (ed.), Classical swine fever and related viral infections. Martinus Nijhoff Publishing, Boston, MA.
52. **Wiskerchen, M., S. K. Belzer, and M. S. Collett.** 1991. Pestivirus gene expression: the first protein product of the bovine viral diarrhea virus large open reading frame, p20, possesses proteolytic activity. J. Virol. **65:**4508–4514.
53. **Yoneyama, M., W. Suhara, Y. Fukuhara, M. Sato, K. Ozato, and T. Fujita.** 1996. Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). J. Biochem. (Tokyo) **120:** 160–169.
54. **Zhu, Q., H. Yang, W. Chen, W. Cao, G. Zhong, P. Jiao, G. Deng, K. Yu, C. Yang, Z. Bu, Y. Kawaoka, and H. Chen.** 2008. A naturally occurring deletion in its NS gene contributes to the attenuation of an H5N1 swine influenza virus in chickens. J. Virol. **82:**220–228.